Molecular characterisation of Staphylococcus aureus strains isolated from small and large ruminants reveals a host rather than tissue specificity.

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P. D. D. Alves[1,2,3§], J. A. McCulloch[1,2§], S. Even[1,2], C. Le Maréchal[1,2], A. Thierry[1,2], N. Grosset[1,2], V. Azevedo[3], C. A. Rosa[3], E. Vautor[4] and Y. Le Loir[1,2]*

(1) INRA, UMR1253 STLO, 85 rue de Saint Brieuc, 35042 Rennes, France;
(2) Agrocampus Ouest, UMR1253 STLO, 85 rue de Saint Brieuc, 35042 Rennes, France;
(3) Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Brazil;
(4) PPR, AFSSA Sophia Antipolis, France

[§]: equal contribution
*: corresponding author
yves.leloir@rennes.inra.fr

Abstract

Staphylococcus aureus is an important pathogen in domestic ruminants. The main objective of this study was to determine the similarity of epidemiologically unrelated *S. aureus* isolates from bovine, ovine, and caprine hosts regardless the locus of isolation (nasal carriage, udder skin or mastitis milk). By pulsed-field gel electrophoresis, 7 major pulsotypes were identified among 153 isolates recovered from 12 different regions of France. Typing of the accessory gene regulator (*agr*) and capsular (*cap*) serotype was carried out on all the isolates and revealed the predominance of *agr*I and III and of *cap8* regardless the ruminant host species. Antimicrobial susceptibility testing revealed resistance to ampicillin in 34% of strains. A very low prevalence of resistance was found for the other antimicrobial agents tested (kanamycin, tetracyclin and oxacillin). These results suggest the existence of a host rather than tissue specificity among *S. aureus* isolates colonising the ruminant species and suggest a limited transmission of those isolates between large (bovine) and small (ovine-caprine) ruminants. The *agr* class and *cap* types correlated with pulsotype clusters rather than with a specific host species. Antimicrobial resistance appears not to have contributed to the predominance of any given genotypes, and MRSA prevalence appears very low in ruminant isolates.

Keywords; *Staphylococcus aureus*, host specificity, genotype, resistance to antibiotics

1. Introduction

S. aureus is one of the most frequent etiologic agents of mastitis in bovines, ovines and caprines, rendering livestock unable to adequately produce milk, which results in heavy economic losses for the dairy industry (Seegers et al., 2003). Prevention and treatment of mastitis remains a major concern for veterinary science. Studies characterising ruminant isolates of *S. aureus* are scarce compared to the much more abundant data on human isolates found in the literature. Ruminant *S. aureus* isolates have been reported as being distinct from human ones. Devriese et al. first described phenotypic differences between *S. aureus* strains isolated from humans and other animal hosts including bovines and ovines, and proposed a scheme for the distinction of several host biotypes (Devriese and Oeding, 1976). Differences between host biotypes are also reflected at the genotypic level as determined by macro-restriction analysis of the chromosome (Hennekinne et al., 2003). Due to the specificity of host-pathogen interactions needed to produce mastitis, it has been postulated that the nature of the virulon and the regulation of its expression are determining factors when it comes to the ability of a strain to produce mastitis (Vautor et al., 2008). The recent release of the complete genome sequence of *S. aureus* ET-3, a bovine isolate, provides new insight into the genomic basis of a putative host adaptation and the existence of host specific genetic traits in *S. aureus* isolated from bovine hosts (Herron-Olson et al., 2007). We recently showed that diversification of the *S. aureus* core genome correlated with host origin in ruminants (Ben Zakour et al., 2008). However, in most studies, the panel of ruminant *S. aureus* strains comprised mostly strains isolated from cases of mastitis. The mammary gland tissues present characteristics such that some authors hypothesized that the specific traits found to be common in bovine strains were related to a tissue- rather than to host- specificity (van Leeuwen et al., 2005). In the present study, we characterised several *S. aureus* isolates obtained from different sites of colonisation / infection, from ruminants in different geographic regions, by pulsed-field gel electrophoresis (PFGE), and further characterised the

strains as to their *agr* group and capsular polysaccharide genotype, as well as to their susceptibilities to antimicrobial agents. The aims of this characterisation were to verify whether the strains groups reflected a host- or tissue-adaptation, whether there is a predisposition of certain *cap* or *agr* types to colonise or infect certain ruminant hosts, and to evaluate the spread of resistance to methicillin and to the most commonly used antibiotics in the treatment of mastitis in ruminants.

2. Material and Methods

2.1. Bacterial strains.

A total of 153 *S. aureus* isolates were either chosen from existing collections or were collected specifically for this study. Most isolates from bovine (65 strains), ovine (57), and caprine (31) hosts were collected between 1964 and 2006 in France (117), and Brazil (30). The panel of strains included also isolates from Belgium (5) and the USA (1). The strains collected in this study were isolated from the udder (mastitic milk and udder skin) and nares of bovine, ovine, and caprine hosts as described previously (Vautor et al., 2003). Samples were first grown on selective Baird-Parker medium. Species identification was carried out on coagulase positive staphylococci using previously described PCR tests (Baron et al., 2004;Morot-Bizot et al., 2004). Details of the sampling used in this study (locus of isolation and host) are given in Table 1.

2.2. PFGE. Pulsed-Field Gel Electrophoresis for the characterisation of the strain lineage was carried out according to previously described protocols (Prevost et al., 1992b). Briefly, cells from a pure culture were lysed in agarose blocks by incubation at 37°C, for 4h in TE buffer (Tris 10 mM, EDTA 0.5 M, pH 8) supplemented with lysostaphin (25 µg/mL). DNA was digested with 20U of *Sma*I for 18h at 25°C. The resulting fragments were submitted to pulsed-field electrophoresis using the CHEF DRII system (Biorad) with the following

parameters: 200V, an initial pulse of 2s, final pulse of 20s and 20h of migration at 14°C. The band profiles obtained for each strain were analysed using the Bionumerics software, version 2.0 (Applied-Maths, Belgium).

2.3. Determination of the *agr* group. Classification of strains into *agr* interference groups was carried out by PCR according to Gilot *et al.* (Gilot and van Leeuwen, 2004), which involves a forward primer common to all *agr* groups and four primers, each one specific to each *agr* group. All PCRs were run with the following conditions: a hot start of 95 °C for 5 min, followed by 30 cycles of 95°C for 1 min, 56°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 7 min.

2.4. Determination of the capsular polysaccharide type. The capsular polysaccharide type was determined by means of PCR with primers directed to sequences of the staphylococcal capsular polysaccharide gene (*cap*) specific to either the type 5 (primers Cap5k1 and Cap5k2) or type 8 (primers Cap8k1 and Cap8k2) alleles, as described by Verdier et al. (Verdier et al., 2007). Each strain was submitted to a reaction using either the Cap5k1 and Cap5k2 or the Cap8k1 and Cap8k2 primer pair in independent reactions. Reactions were carried out with the following conditions: a hot start of 95 °C for 5 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 7 min.

2.5. Determination of the susceptibility of strains to antibiotics. Susceptibilities of strains to ampicillin, oxacillin, kanamycin and tetracycline were evaluated by means of the Kirby-Bauer disk diffusion method as described previously (CLSI, 2007).

2.6. Statistical analyses.

A κ^2 test was used to determine the significance of occurrence of genes in a host specific group by use of Statgraphics version 5.1. The nominal P value for statistical significance was 0.05.

3. Results and discussion

3.1. *S. aureus* strains grouped with regard to their host- but not tissue- origin.

Macrorestriction profile analysis using PFGE is one of the most discriminative methods for *S. aureus* when compared to other genotyping methods (Prevost et al., 1992a;van Belkum et al., 1994;Kuhn et al., 2007). All the strains studied here grouped up into 7 clusters (A to G – figure 1) when analyzed by PFGE considering a similarity cut-off of 48 %. Each cluster was significantly correlated (P>0.05) to the subfamily of the host (bovine for large ruminants or ovine-caprine for small ruminants) from which the strains were isolated. Three clusters were majorly composed of bovine isolates: 42 out of 45 strains (93 %) are bovine isolates in cluster B; 7/10 strains (70 %) in cluster C; and 7/13 strains (54 %) in cluster E. The four other clusters comprised a great majority of small ruminants isolates: 24 out of 24 strains (100 %) are ovine-caprine isolates in cluster A; 43/49 strains (88%) in cluster D; 7/9 strains (78 %) in cluster F; and 3/3 strains (100 %) in cluster G. Within the small ruminant isolates, strains from ovine- and caprine origins could not be distinguished based on the pulsotype. They were evenly distributed among pulsotype clusters A, D, F, and G. Strains isolated from ovine and caprine hosts presented a similarity of up to 100 %, whereas no bovine strain presented a 100% similarity to a small ruminant strain. Previous studies based on CGH analysis of *S. aureus* isolates including a few bovine strains suggested the existence of tissue- rather than host- specific genetic traits (van Leeuwen et al., 2005). However, in these studies, only a few bovine mastitis isolates were analyzed amongst a large panel of human strains. In the present study, we included strains isolated from the nares and udder of various ruminant hosts and results showed that they were evenly distributed amongst each of the clusters (Figure 1). Thus *S. aureus* strains clearly grouped up into clusters which correlated to the type of host they were isolated from, regardless the locus of isolation. Our results are in contrast to another work showing that there is little or no host preference among *S. aureus* presenting different

genotypes (Mork et al., 2005), in which isolates were obtained from milk collected from cases of clinical and subclinical mastitis. On the other hand, our results are in accordance with previous studies which reported that host biotypes, as determined according to Devriese's scheme, are reflected by PFGE profiles (Hennekinne et al., 2003). Lineages might be adapted to colonise a certain type of host independent of the site of colonisation/infection. The fact that there was significant correlation between the sub-family of the animal host and lineage, in spite of the strains having been isolated from very different geographical locations (all major regions of France, plus isolates from Brazil, Belgium, and the USA) and in different years (from 1964 to 2006), strongly indicates that a certain strain has a penchant for the type of host it is successful in living on.

3.2. Determination of *agr* group. The ability of *S. aureus*, as a species, to endure in different niches, playing different roles in the relationship with an animal host is testament to either the regulation of the expression of a panoply of accessory genes, or a variability of strains within the species, with lineages presenting a collection of genes specializing in a particular *modus vivendi* (Ben Zakour et al., 2008;Fitzgerald and Musser, 2001). The expression of the accessory genes in *S. aureus* is under the control of a series of systems that interact with each other to form a network. Of all these systems, the accessory gene regulator (*agr*), a two-component quorum-sensing system has arguably been the most studied in *S. aureus*. Four interference classes related to genetic polymorphisms in the *agr* locus have thus far been described, namely, *agr* groups I, II, III and IV (Jarraud et al., 2002a;Ji et al., 1995). So far, *agr* variability has been only rarely studied in isolates obtained from ruminants (Gilot and van Leeuwen, 2004;Reinoso et al., 2008;Vautor et al., 2008).

In this study, altogether, the most prevalent *agr* group found was *agr* I (n=91, 59.5%), followed by *agr* III (n=40, 26.1%) and *agr* II (n=21, 13.7%). Only a single strain bearing an *agr* IV polymorphism was found (0.6%) and corresponded to a bovine isolate. If stratified by host type (small or large ruminant), within bovines, the prevalence of *agr* groups I, II, III and

IV were 54.7%, 17.2%, 26.6% and 1.6%, respectively, whereas within small ruminants, their prevalence was 62.9%, 11.2%, 25.8% and 0%. The ratios of the prevalences of each *agr* group are approximately the same when considering only small or only large ruminants. This suggests that *agr* type does not play a role in host specificity. When considering discrete genotype clusters, it appears that, for a given host, some genotypes have a high prevalence of *agr* I whereas for some others *agr* III is clearly prevalent. It has been previously shown that *S. aureus* strains belonging to *agr* group I have a greater ability of invading epithelial cells and persist in the mammary gland, respectively, suggesting that they are better at causing clinical or subclinical mastitis than strains of other *agr* groups (Buzzola et al., 2007). Indeed, this same study found a disproportionately high prevalence (88%) of *agr* type I strains causing mastitis in bovines. Our study shows that *agr* group distribution among ruminant isolates correlated with genotype cluster rather than with a given host. Of note, the distribution of *agr* groups was tissue-independent since isolates from mastitis milk, udder skin or nares were found in each cluster. This observation is in agreement with Jarraud et al. (Jarraud et al., 2002b), who demonstrated a relationship between the genetic background of the strains and the *agr* allele group. We previously reported the prevalence of *agr* III (46% vs 44% for *agr* I) in small ruminant isolates and a correlation between geographical region and predominance of a given *agr* group was hypothesized to explain predominance in some lineages (Vautor et al., 2008). Here, strains originating from various geographical regions grouped within the same clusters and belonged to the same *agr* group. This suggests that the link between *agr* allele groups and a given region is likely due to the predominance of a given lineage within the region considered.

3.3. Determination of the capsular polysaccharide type. The *cap* operon responsible for the biosynthesis of capsular polysaccharides (CP) expressed on the surface of *S. aureus* belongs to the virulon (O'Riordan and Lee, 2004). There are 11 serotypes of CP, however, the

majority of strains isolated from humans express either CP type 5 or type 8, for which PCR a test has been developed (O'Riordan and Lee, 2004;Verdier et al., 2007). In contrast, a variable prevalence of different CP types has been observed in strains isolated from ruminants from geographically different regions of the world (Guidry et al., 1997;Poutrel et al., 1988;Sordelli et al., 2000) and the actual role of CP in mastitis is questioned (Tuchscherr et al., 2005). Here, strains were CP typed using a PCR test enabling differentiation between CP type 5 and 8. The prevalence of the types of CP (5, 8 or non-typeable) for each lineage can be seen in Figure 1. Altogether, the capsular type 8 was predominant in this study and accounted for 65.4 % of the panel. CP type 5 and the non-typeable CP types accounted for 30.7 % and 3.9 % respectively. When considering the host species, CP type 8 was clearly predominant in small ruminants, with an overall prevalence of 83.1 % in ovine-caprine isolates. In contrast, CP type 5 was slightly predominant in bovine isolates (56.3 %) and the prevalence of one type over the other seemed dependent of the genotype cluster. The bovine clusters B and C were indeed majorly CP type 5. These results are in accordance with previous studies on CP typing of *S. aureus* mastitis isolates showing that most bovine isolates were CP type 5 whereas ovine and caprine isolates were CP type 8 (Guidry et al., 1997;Poutrel et al., 1988). The low prevalence of non-typeable strains in this study contrasted with previous work where up to 76.5% of bovine mastitis strains (Sompolinsky et al., 1985) were found to be non-typeable. However, in this latter study only 17 strains were typed and the geographical region of isolation is not mentioned although this criterion, as shown above, is of great importance and may, in this case, bias the results.

3.4. Susceptibility to antibiotics. The control of the spread of *S. aureus* has become challenging due to this species' ability to resist to antimicrobial therapy, taking into account that it has vanquished almost every existing currently available antimicrobial agent (Hiramatsu et al., 1997). The prevalence of antibiotic resistance among strains isolated from domestic animals is increasing, raising concerns about the role of domestic animals as

reservoirs of *S. aureus* which may become involved in human infections (Anderson et al., 2008;de Neeling et al., 2007;Khanna et al., 2008). In this study, Kirby-Bauer antibiogrammes showed that resistance to antibiotics was infrequent amongst *S. aureus* strains isolated from ruminants. Interestingly, resistance to tetracycline (n=3, 2.0%) and kanamycin (n=8, 5.2%), two low cost antimicrobials commonly used in mastitis treatment, were rare. Resistance to the beta-lactam ampicillin was the most prevalent with 52 strains (34.0%) displaying this phenotype. This result is in accordance with the high prevalence of beta-lactamase (*bla*) genes in community strains (Maranan et al., 1997). Only 5 strains (3.2%) presented resistance to oxacillin, an antibiotic used for the detection of Methicilin Resistant *S. aureus* (MRSA). Our findings contrast with recent studies in which MRSA was found in the nares of 39% and 10.9% of pigs and horses tested, respectively (de Neeling et al., 2007;Van den Eede A. et al., 2008). Together with other studies (Juhasz-Kaszanyitzky et al., 2007), our results confirmed that MRSA prevalence is still low in ruminants.

4. Conclusions

S. aureus strains are phenotypically and genomically variable. Pulsotyping of ruminant isolates showed that *S. aureus* strains clustered with regard to their host origin (bovine and ovine-caprine) and regardless the locus of colonization or infection. Accessory gene regulator polymorphism and the type (or presence) of capsular polysaccharide are facets of strain-to-strain difference. The prevalence of each *agr* group and *cap* type within each lineage varied with the lineage, however, when analysed within the context of each sub-family of host (small or large ruminant), the prevalences of each *agr* group or *cap* type are about the same either in bovines, or ovines/caprines. This hints at the hypothesis that adaptation to a host is not a result of the regulation of a set of accessory genes, but rather the presence/absence or allelic variation of certain genes. The fact that stains found in small ruminants are different from those found in bovines is important enough to warrant the research and development of different immunoprophylactic products against mastitis in small and large ruminants. The low

prevalence of resistance to antibiotics found in ruminants indicates that domesticated livestock is not a reservoir of genes which provide resistance to antimicrobial drugs.

In conclusion, this study shows that the nature of *S. aureus* strains differs between large and small ruminants and suggests the existence of a host rather than tissue specificity. Further studies are being pursued for the determination of the molecular nature of this host specificity and strategies for mastitis prevention in small or large ruminants should take account of these differences.

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Table 1 – *S. aureus* isolates used in this study.

Sample site	**No. of isolates**		
	Bovine	**Caprine**	**Ovine**
nares	14	5	39
mammary skin	0	2	0
mastitis	22	14	17
milk	29	10	1
Total	65	31	57

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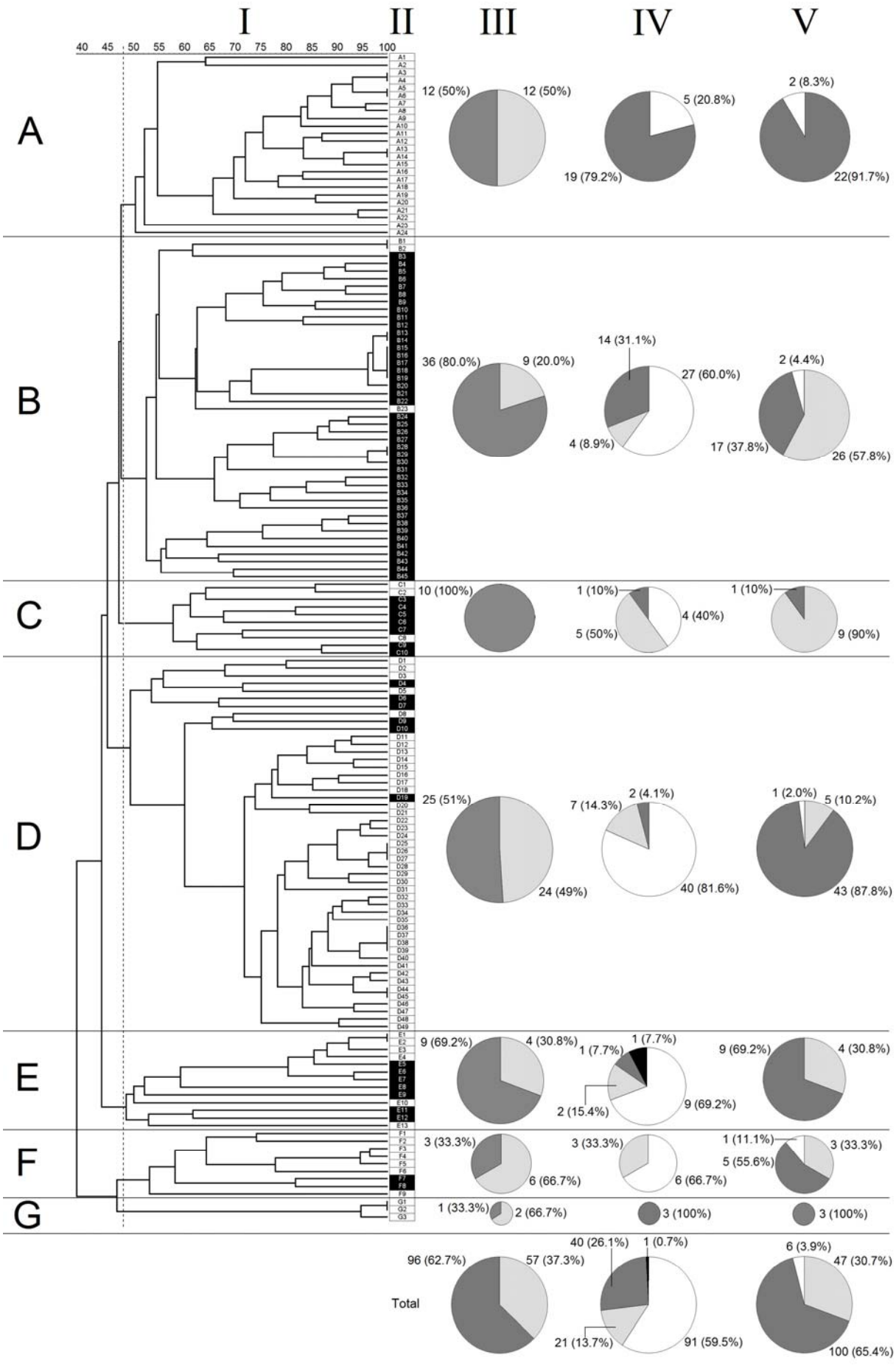
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5 (20.8%)
19 (79.2%)
2 (8.3%)
22(91.7%)
36 (80.0%)
9 (20.0%)
14 (31.1%)
27 (60.0%)
4 (8.9%)
2 (4.4%)
17 (37.8%)
26 (57.8%)
10 (100%)
1 (10%)
4 (40%)
5 (50%)
1 (10%)
9 (90%)
25 (51%)
24 (49%)
7 (14.3%)
2 (4.1%)
40 (81.6%)
1 (2.0%)
5 (10.2%)
43 (87.8%)
9 (69.2%)
4 (30.8%)
1 (7.7%)
1 (7.7%)
2 (15.4%)
9 (69.2%)
9 (69.2%)
4 (30.8%)
3 (33.3%)
6 (66.7%)
3 (33.3%)
6 (66.7%)
1 (11.1%)
3 (33.3%)
5 (55.6%)
1 (33.3%)
2 (66.7%)
3 (100%)
3 (100%)
Total
96 (62.7%)
57 (37.3%)
40 (26.1%)
1 (0.7%)
21 (13.7%)
91 (59.5%)
6 (3.9%)
47 (30.7%)
100 (65.4%)

Figure 1. PFGE genotyping of the 153 S. aureus strains isolated from ruminants and identification of their *agr, cap* group. I, Dendrogram depicting the PFGE macrorestriction analysis of the chromosome and presenting the percentage of genetic similarity between the 153 strains. The unweighted-pair group method using average linkages and a Dice coefficient (with a tolerance limit of 1%) were used to build the dendrogram. A dashed line indicates the cut-off value (48%) chosen to determine the 7 clusters indicated A to G. II, Host of origin of the strains (black, bovine isolates; white, small ruminant isolates). III, site of isolation of the strains belonging to each of the 7 PFGE clusters identified (light grey, nares; dark grey, udder). IV, distribution of *agr* groups within each of the 7 PFGE clusters (white, *agr* I, light grey, *agr* II, dark grey, *agr* III, black, *agr* IV). V, distribution of *cap* groups in the 7 clusters (light grey, *cap5*, dark grey, *cap8*; white, non-typeable). The last row gives the overall proportions for site of isolation, *agr* and *cap* groups within the entire panel of strains.